

Effects of Antibodies against Dynein and Tubulin on the Stiffness of Flagellar Axonemes

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ABSTRACT Antidynein antibodies, previously shown to inhibit flagellar oscillation and active sliding of axonemal microtubules, increase the bending resistance of axonemes measured under relaxing conditions, but not the bending resistance of axonemes measured under rigor conditions. These observations suggest that antidynein antibodies can stabilize rigor cross-bridges between outer-doublet microtubules, by interfering with ATP-induced cross-bridge detachment. Stabilization of a small number of cross-bridges appears to be sufficient to cause substantial inhibition of the frequency of flagellar oscillation.

Antitubulin antibodies, previously shown to inhibit flagellar oscillation without inhibiting active sliding of axonemal microtubules, do not increase the static bending resistance of axonemes. However, we observed a viscoelastic effect, corresponding to a large increase in the immediate bending resistance. This immediate bending resistance increase may be sufficient to explain inhibition of flagellar oscillation; but several alternative explanations cannot yet be excluded.

Antibodies against flagellar proteins have been developed for use as probes of flagellar function, in a effort to obtain new insight into the mechanisms of flagellar bending. The feasibility of this approach depends on the use of demembranated flagella which, in addition to being accessible to antibodies, show nearly normal motility when reactivated in solutions containing ATP. The first studies used an antibody prepared against a tryptic fragment of sea urchin flagellar dynein, Fragment A (18). This antibody was shown to inhibit the ATPase activity of Fragment A (18), native dynein 1 (18), and intact axonemes (17). It inhibited the motility of demembranated sea urchin sperm flagella (14, 22) by decreasing the beat frequency (14) and, in some cases, the bend angle (17). This antibody was also shown to inhibit sliding between flagellar microtubules, when assayed by observing ATP-induced sliding disintegration of axonemes previously digested with trypsin (15) or elastase (1). An antibody against native dynein 1 was prepared by us (17) and shown to have similar, but weaker, effects on the motility of demembranated flagella. This antibody bound to dynein but did not measurably inhibit its ATPase activity.

Asai and Brokaw (1) examined the effects of several antibodies against tubulin, obtained after immunizing rabbits with various types of tubulin preparations from sea urchin sperm flagella and purifying the antibodies by tubulin-affinity chromatography. All of these antibodies specifically reduced the bend angle of motile demembranated sea urchin sperm flagella, without inhibiting beat frequency, but they differed in their

potencies. Under conditions where the most potent antitubulin gave a complete inhibition of flagellar bending, there was no inhibition of the sliding disintegration of elastase-digested axonemes. These observations demonstrated convincingly that antibodies against different flagellar proteins can alter flagellar function in distinctly different ways.

Measurements of the stiffness, or bending resistance, of flagellar axonemes provide an additional source of information about the interactions between flagellar components. Stiffness measurements on demembranated sea urchin sperm flagella have identified two distinct states: a relaxed state of relatively low stiffness and a rigor state of relatively high stiffness (20, 21). These are interpreted as states of low and high degrees of attachment of dynein arms to B-tubules to form stable cross-bridges. The existence of a stable rigor state can also be demonstrated by the preparation of flagella showing stable rigor bends (13). Here we measure the stiffness of flagellar axonemes in the presence of antibodies against dynein or tubulin and interpret the results in relation to the other observations on the effects of these antibodies.

MATERIALS AND METHODS

These measurements were carried out with spermatozoa from the sea urchin, *Strongylocentrotus purpuratus*, the same species that provided the tubulin preparations used to obtain the antibodies against tubulin. Demembranated spermatozoa were prepared by the procedure of Brokaw et al. (6), using 5 mM CaCl₂ in the Triton X-100 extraction solution with the minor modification of diluting the

spermatozoa 1:10 with 0.5 M NaCl for storage at 0°C and then diluting them 1:20 in the Triton X-100 extraction solution. Demembrated spermatozoa were then diluted into a working solution consisting of working solution base (WS) plus other constituents as required. WS contained: 0.2 M KCl, 1.8 mM MgCl₂, 1.8 mM EGTA, 0.5 mM CaCl₂, 2 mM dithiothreitol, 20 mM Tris-Cl, and 2% (wt/vol) polyethylene glycol, with the pH adjusted to 8.2. The standard procedure for experiments involving incubation with antiserum was the following: 5 μ l or less of demembrated sperm suspension was mixed with 0.40 ml of WS made up with 10/9 of the final concentrations. This was then mixed with 50 μ l of antibody solution which had been dialyzed overnight against WS. After 30-min incubation on ice, the suspension was mixed with 50 μ l of a solution containing 10 mM MgCl₂ and 11 mM ATP. When necessary, vanadate (sodium orthovanadate, Na₂VO₄) was added to a final concentration of 10 μ M, to inhibit flagellar movement.

Stiffness measurements were carried out with the technique described previously (20) with one modification: in previous experiments the sperm head was fixed to a polylysine-coated microneedle to support the proximal end of the flagellum. In our experiments, spermatozoa were selected in which the proximal portion of the flagellum adjacent to the head, ~5 μ m long, was also fixed to the flat, polylysine-coated microneedle, as illustrated in Fig. 4. Techniques for observation by dark-field microscopy and measurement of beat frequency by stroboscopic illumination were the same as in previous work (1, 20). All measurements were made at 18°C.

Antibody Preparations

The antitubulin used in these experiments was the one identified as r5 in the work of Asai and Brokaw (1). It was prepared using twice KCl-extracted, and low ionic strength-extracted flagellar axonemes from *S. purpuratus* as an antigen and then purified by tubulin-affinity chromatography using electrophoretically purified outer-doublet tubulin from *S. purpuratus*.

The antidynein used in these experiments was the one described by Ogawa, Negishi, and Obika (19). It was prepared using dynein 1 purified from KCl extracts of axonemes of spermatozoa from the sea urchin *Anthodidaris crassispina*. The IgG fraction was prepared according to standard methods, employing a one-third saturated ammonium sulfate precipitation and DEAE cellulose chromatography (10). This antibody was found to inhibit the ATPase activity of dynein 1 and Fragment A of dynein 1 (19) and is therefore closely related in its properties to the anti-Fragment A serum used previously.

RESULTS

Control Experiments

The flagella of *S. purpuratus* spermatozoa are 15–20% shorter than those of *Lytechinus pictus* used in earlier stiffness measurements (5). In addition, the attachment of about 5 μ m of the proximal end of the flagellum to the polylysine-coated microneedle further reduced the length of the flagellum used for the stiffness measurement, to ~20 μ m. Nevertheless, the values obtained for the stiffnesses of the relaxed and rigor states (0.8×10^{-21} N m² and 11×10^{-21} N m², respectively, Table I) of *S. purpuratus* sperm flagella are very close to the values obtained previously with *L. pictus* spermatozoa.

Flagella incubated for 30 min with nonimmune IgG (1 mg/ml) had stiffness values indistinguishable from those of flagella measured in the absence of antibody, in both the rigor and relaxed states, as shown in Table I, lines 1–6. After incubation for 30 min with nonimmune IgG (1 mg/ml) in the absence of MgATP²⁻, normal motility was observed on adding 1 mM MgATP²⁻, and most of the spermatozoa remained motile for an hour or more. Fig. 1 shows results of measurements of beat frequency of flagella in the presence of nonimmune IgG (200 μ g/ml). There is no change in beat frequency during the 10-min period covered by these measurements.

Effects of Antidynein Antibodies

Fig. 1 shows the effect of antidynein IgG (200 μ g/ml) on the beat frequency of demembrated flagella in the presence of 1 mM MgATP²⁻. During the 10-min period covered by these measurements, there was a rapid decrease in beat frequency,

TABLE I
Stiffness of Flagella

Incubation 30 min at 0°C	Reagents added after the incubation	Stiffness (\pm SD) (10^{-21} N m ²)	No. of spermatozoa measured
(1) WS	MgATP ²⁻ , vanadate	0.84 ± 0.13	8
(2) WS, MgATP ²⁻ , vanadate	None	0.76 ± 0.13	7
(3) WS, non-immune IgG (1 mg/ml)	MgATP ²⁻ , vanadate	0.94 ± 0.18	4
(4) WS, non-immune IgG (1 mg/ml), MgATP ²⁻ , vanadate	None	0.63 ± 0.08	5
(5) WS	None	11.1 ± 1.4	4
(6) WS, non-immune IgG (1 mg/ml)	None	12.0 ± 0.7	5
(7) WS, antidynein (200 μ g/ml)	MgATP ²⁻	5.2 ± 0.9	5
(8) WS, antidynein (200 μ g/ml)	MgATP ²⁻ , vanadate	5.3 ± 1.0	10
(9) WS, antidynein (200 μ g/ml), MgATP ²⁻ , vanadate	None	3.4 ± 1.0	9
(10) WS, antidynein (200 μ g/ml)	None	12.4 ± 3.9	14
(11) WS, antitubulin (50 μ g/ml)	MgATP ²⁻	0.88 ± 0.29	6
(12) WS, antitubulin (50 μ g/ml)	MgATP ²⁻ , vanadate	0.88 ± 0.18	10
(13) WS, antitubulin (100 μ g/ml)	MgATP ²⁻ , vanadate	0.87 ± 0.12	2
(14) WS, MgATP ²⁻ , vanadate, antitubulin (50 μ g/ml)	None	1.02 ± 0.21	4
(15) WS, antitubulin (50 μ g/ml)	None	8.6 ± 0.5	2

which was accompanied by a decrease in bend angle. After 10 min, none of the flagella continued to beat regularly, but many showed irregular beating, with head oscillation and intermittent bend propagation. Within 20 min, all of the flagella stopped completely. Incubation with antibody in the absence of ATP appeared to be even more effective; after 7-min incubation on ice, none of the flagella were reactivated with 1 mM MgATP²⁻.

Rigor-wave flagella prepared according to the method of Gibbons and Gibbons (13) and then incubated with antidynein IgG (200 μ g/ml) for 30 min on ice maintained rigor bends both before and after addition of 1 mM MgATP²⁻, as previously shown by Gibbons et al. (14), in which an anti-Fragment A serum was used. Incubation with antidynein IgG (200 μ g/ml) had no significant effect on the stiffness of flagella in the rigor state, as shown in Table I, line 10.

Fig. 2 shows the results of experiments in which the stiffness of demembrated flagella was measured during incubation with antidynein IgG (200 μ g/ml) in the presence of 1 mM MgATP²⁻ and 10 μ M vanadate. Data for five different flagella are shown. At intervals of 3 or 5 min, three or more measurements were quickly made on each flagellum to determine the stiffness. The results show a gradual increase in stiffness from the value characteristic of the relaxed state to a value about four times greater after 30 min of incubation. No further increase in stiffness could be detected after further incubation.

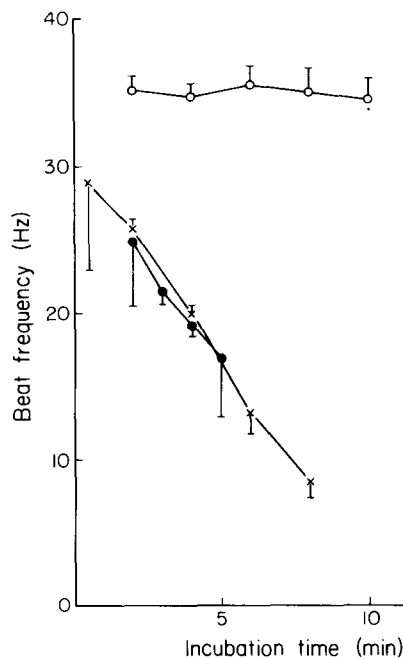


FIGURE 1 Measurements of beat frequency of flagella of spermatozoa attached by their heads to the microscope slide, at 1 mM ATP, in the presence of non-immune IgG (200 μ g/ml) (○) or in the presence of antidynein antibodies (200 μ g/ml) (x, ●). Two groups of flagella were measured in the presence of the antidynein antibodies. Each group included more than ten spermatozoa observed in the field of view obtained with a $\times 16$ microscope objective. After incubation with antidynein antibodies for 5 min, the number of immobilized and irregularly beating flagella increased. Measurements were made on those flagella that retained a regular beat. Vertical bars are standard deviations.

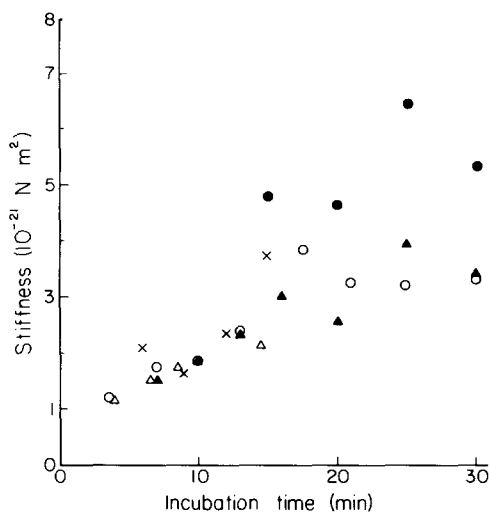


FIGURE 2 Change in stiffness of flagella during incubation with antidynein antibodies (200 μ g/ml) in the presence of 1 mM ATP and 10 μ M vanadate. Each point represents three or more measurements in one direction. The symbols identify the five different flagella that were measured.

In other experiments, demembranated spermatozoa were incubated with antidynein IgG for 30 min on ice in the presence of MgATP²⁻ and vanadate, and then measured. In these cases, the mean stiffness was similar (3.4×10^{-21} N m², Table I, line 9) to the result shown in Fig. 2.

Flagella measured in the presence of MgATP²⁻, with or

without vanadate, after incubation with antidynein IgG on ice in the absence of MgATP²⁻ and vanadate, had somewhat higher values of stiffness (5.2×10^{-21} N m²), about half the rigor value (Table I, lines 7 and 8).

By photographing the position of the flagellum during stiffness measurements at 2-s intervals after moving the measurement microneedle against the flagellum, it was possible to measure the approach to the equilibrium position that is used to determine the stiffness of the flagellum. In the control experiments shown in Fig. 3, the initial position of the flagellum photographed at 2 s corresponds to a slightly higher stiffness (1.15×10^{-21} N m²) than the final stiffness (0.8×10^{-21} N m²) that characterizes the relaxed state. The initial stiffness is ~44% greater than the final value. This can be described as viscoelastic behavior of the flagellum. A similar small viscoelastic effect is also shown in Fig. 3 for the measurements of stiffness in the presence of antidynein antibodies, with the initial stiffness being ~16% higher following incubation with antidynein antibodies in the absence of MgATP²⁻ and ~80% higher following incubation with antidynein antibodies in the presence of MgATP²⁻.

Effects of Antitubulin Antibodies

Complete inhibition of flagellar bending was obtained with the antitubulin IgG (50 μ g/ml) in the presence of MgATP²⁻ after 30-min incubation with the antibody on ice. As shown in Table I, antitubulin IgG (50 μ g/ml or 100 μ g/ml) had no effect on the static stiffness of the relaxed state, in the presence of 1 mM MgATP²⁻, with or without vanadate, and had no signif-

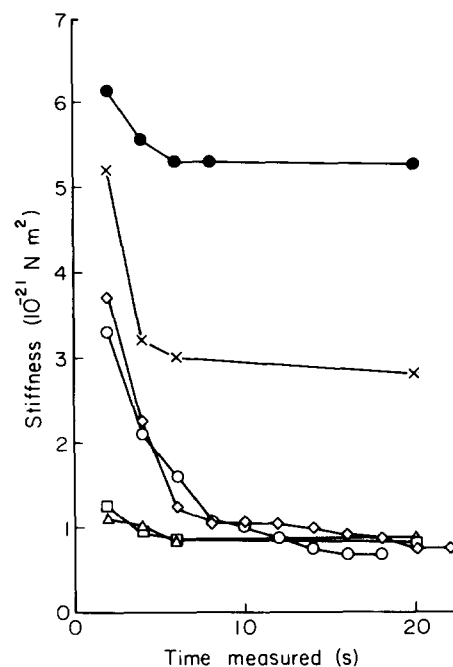


FIGURE 3 Change in apparent stiffness of flagella with time. Photographs taken at 2-s intervals, as shown in Fig. 4, were used to calculate the apparent stiffness at each time-point. Each curve represents measurements on one flagellum, under the following conditions: (●) 1 mM ATP and 10 μ M vanadate, after incubation with antidynein antibodies (200 μ g/ml). (x) Incubation with antidynein antibodies in the presence of 1 mM ATP and 10 μ M vanadate. (◇, ○) 1 mM ATP and 10 μ M vanadate, after incubation with antitubulin antibodies (50 μ g/ml). (□) 1 mM ATP and 10 μ M vanadate, after incubation with non-immune IgG (1 mg/ml). (Δ) 1 mM ATP and 10 μ M vanadate, after incubation without antibodies.

icant effect on the stiffness of the rigor state obtained in the absence of MgATP^{2-} .

In contrast to the results with antidynein, there is a very large viscoelastic effect when the stiffness is measured following incubation with antitubulin antibodies. The photographs in Fig. 4 illustrate this behavior. The flagellum bent gradually when the microneedle was applied and took ~ 20 s to reach the equilibrium position. After removal of the measurement microneedle, the recovery to a straight position was also gradual, as shown by the lower panels in Fig. 4. The stiffness corresponding to the initial measurement, at 2 s, was $\sim 300\%$ greater than the final stiffness, as shown in Fig. 3.

DISCUSSION

Antidynein Antibodies

Antidynein antibodies have been shown to inhibit flagellar bending and to inhibit sliding between flagellar microtubules. Our stiffness measurements now show that these inhibitory effects are accompanied by an increase in the bending resistance of the axoneme, when measured under relaxing conditions in the presence of MgATP^{2-} and vanadate. Since the antidynein antibodies cause no significant increase in the bending resistance of axonemes that are already under rigor conditions, in the absence of MgATP^{2-} , we suggest that antidynein antibodies inhibit by stabilizing cross-bridges formed by binding of dynein arms to the adjacent B-tubule. Dynein cross-bridge detachment can be induced by MgATP^{2-} , as indicated by the relaxation of rigor-wave flagella by MgATP^{2-} (13) or by measurements of a relaxed stiffness state in the presence of MgATP^{2-} (20). Antidynein antibodies could stabilize cross-bridge attachment by interfering with the access of MgATP^{2-} to a binding site on dynein or by interfering with changes in dynein conformation that are associated with MgATP^{2-} binding and cross-bridge detachment.

When flagella are incubated with antidynein in the absence of MgATP^{2-} , antidynein antibodies may bind to attached cross-bridges and stabilize them in that conformation. Under these conditions, the bending resistance was only about one-half the normal rigor value, suggesting that no more than half of the attached cross-bridges are stabilized by antidynein binding. The simplest explanation of this result might be that antidynein antibodies can only bind to the outer dynein arms, as demonstrated by Ogawa et al. (19). However, it has not been demonstrated that the bending resistance of a rigor axoneme is proportional to the number of rigor cross-bridges.

When flagella are incubated with antidynein antibodies in the presence of MgATP^{2-} , there is initially a strong decrease in flagellar beat frequency and subsequently a measurable increase in bending resistance. Under these conditions, dynein cross-bridges that react with antidynein antibodies presumably fail to bind MgATP^{2-} and detach, and then oppose active sliding in the same manner as cross-bridges that remain attached when the beat frequency is reduced by reducing the MgATP^{2-} concentration. Comparison of the results in Figs. 1 and 2 indicates that the beat frequency can be reduced to $<50\%$ of its initial value under conditions where there is only a very small increase in bending resistance and, presumably, only a small fraction of the dynein arms that has been stabilized by binding antidynein antibodies. A similar result is seen when the MgATP^{2-} concentration is lowered. A reduction in MgATP^{2-} to ~ 0.2 mM is sufficient to reduce the beat frequency to about one-half its maximal value (12) while increases in

bending resistance are not measurable until the MgATP^{2-} concentration is reduced to <10 μM (3). The bending resistance measurements, and other measurements (16, 23), indicate that the MgATP^{2-} concentration for half-maximal binding of MgATP^{2-} to dynein is ~ 1 μM or less. These observations restate a "paradox" observed many years ago—that the ATP concentration required to obtain one-half the maximal beat frequency is much greater than the ATP concentration required for one-half maximal axonemal ATPase activity (2). Apparently, a small number of stable cross-bridges, by failing to bind MgATP^{2-} and detach at an appropriate point in the cross-bridge cycle, can cause a significant retardation in sliding and a major decrease in beat frequency. These cross-bridges may detach without undergoing MgATP^{2-} binding and dephosphorylation cycling if they are stretched sufficiently beyond their equilibrium position (cf. reference 9), but the cross-bridge stretch involved in our measurements of bending resistance is apparently too low to induce this type of detachment.

Stabilization of attached cross-bridges appeared to occur to a lesser extent, or at least more slowly, when flagella were incubated with the antidynein antibodies in the presence of MgATP^{2-} and vanadate, rather than under rigor conditions. If our antidynein antiserum is heterospecific and contains some antibody molecules that react preferentially with a detached dynein conformation, some dynein arms may be immobilized by antidynein binding to a detached form during incubation with MgATP^{2-} and vanadate. These antibody molecules will have no opportunity to bind to dynein if the incubation is carried out under rigor conditions. Alternatively, the inhibitory complex formed by dynein, MgATP^{2-} , and vanadate may be very stable, as has been observed for the inhibitory complex obtained with myosin and vanadate (8, 11), and once formed, antidynein antibodies may be unable to bind and convert this detached dynein state to an attached state. At present, we have no evidence to favor any of these possibilities.

Antitubulin Antibodies

Our previous observations with antitubulin antibodies suggested that these antibodies had a specific effect on the amplitude of flagellar bending waves and inhibited flagellar bending without inhibiting active sliding (1). These effects resembled the effects obtained with CO_2 -inhibition of the motility of demembranated flagella (7). Stiffness measurements on intact sperm flagella immobilized with CO_2 -saturated sea water (21) revealed a relaxed state but did not reveal the viscoelastic behavior associated with the relaxed state obtained with antitubulin antibodies. We have not had an opportunity to determine whether this viscoelastic behavior can be seen with demembranated flagella inhibited with CO_2 under conditions comparable to those in our antitubulin experiments.

Possible interpretations of the combination of effects seen with CO_2 were discussed previously (4). One possibility is that CO_2 and antitubulin antibodies interfere with control mechanisms that may be required to selectively inhibit dynein-tubule interactions in appropriate regions of the axoneme, to produce oscillation and bending wave propagation. Complete inhibition of these control mechanisms would leave the axoneme in a state where all dynein-tubule interactions are enabled, so that any dyneins that are attempting to bend the flagellum will be opposed by dyneins on the opposite side of the axoneme. There would, of course, be no interference with the sliding disintegration of protease-digested axonemes, in which the antagonistic coupling between dyneins on opposite sides of the axoneme is

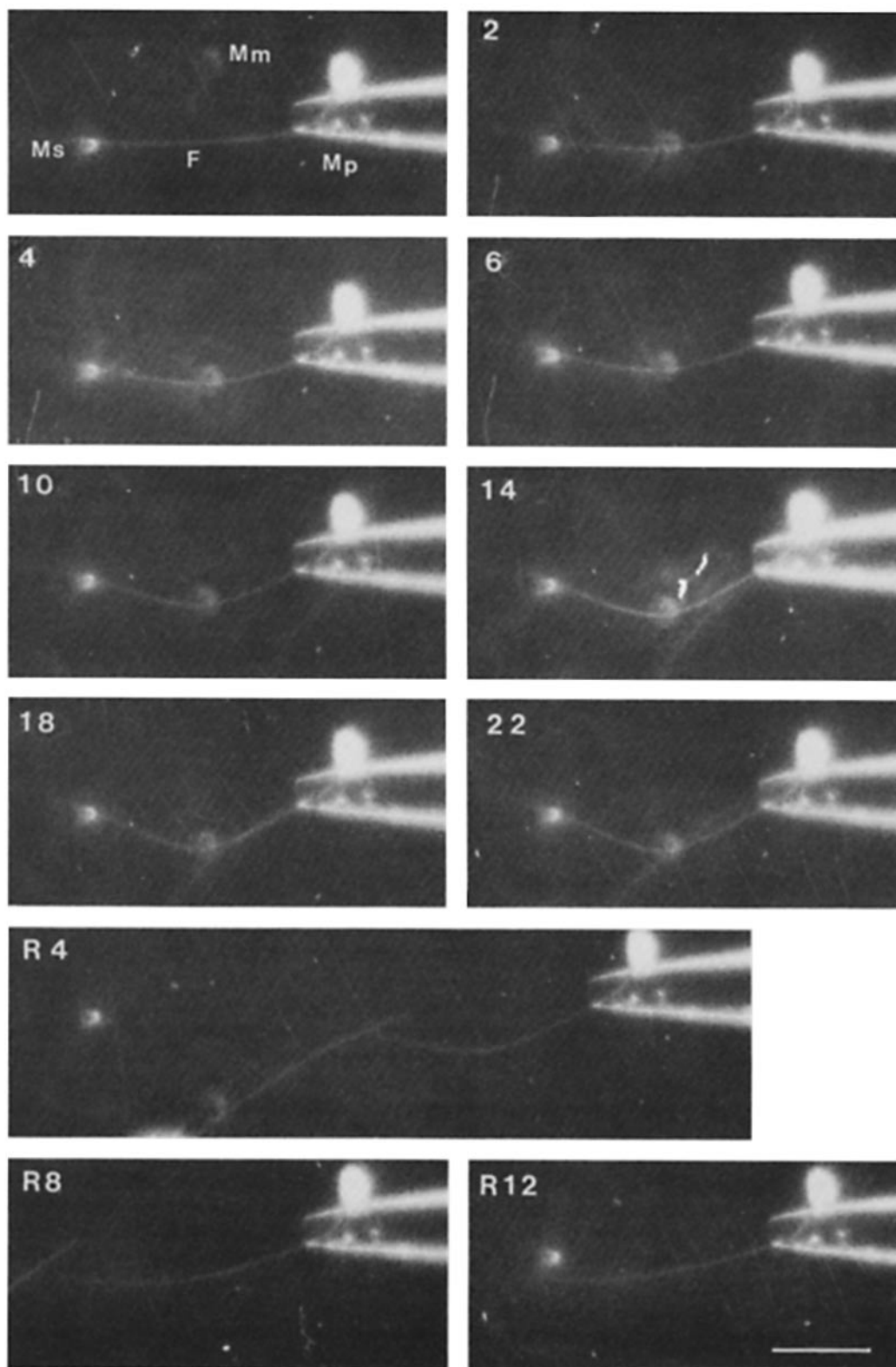


FIGURE 4 Photographic records of a stiffness measurement on a flagellum in 1 mM ATP and 10 μ M vanadate, following 30-min incubation with antitubulin antibodies (50 μ g/ml). The upper eight frames show the change in shape of the flagellum following application of the measurement microneedle, *Mm*. Number on each frame indicates the time in seconds following application of the microneedle. The three lower frames show the recovery of the flagellum at the indicated times (seconds) after removal of the measurement microneedle. *Mp* indicates the polylysine-coated microneedle to which the proximal end of the spermatozoon is attached. *Ms* indicates the microneedle used to support the distal end of the flagellum, *F*. The bar in the bottom right panel indicates 10 μ m.

destroyed. This interpretation is most attractive, because it implies that the viscoelastic behavior observed following incubation with antitubulin antibodies indicates properties of active dynein cross-bridges, which undergo MgATP^{2-} -induced detachment and reattachment to restore the initial state of cross-bridge equilibrium following the distortion imposed by the measurement of bending resistance. If this interpretation is correct, the viscoelastic relaxation time should be very sensitive to MgATP^{2-} concentration, and this should be measurable when more antitubulin antibodies become available.

A second possibility is that antitubulin antibodies and CO_2 prevent effective interaction of dynein with binding sites on the B-tubule, either by blocking these sites or, less directly, by altering the configuration of the outer-doublet microtubules, and thereby decrease the force that can be produced to bend the flagellum. Active sliding disintegration might still appear to be normal, if this is a situation in which the resistance to sliding is negligible, with the sliding rate determined by kinetic properties of dynein cross-bridges. This interpretation does not appear to offer any simple explanation for the viscoelastic behavior we observed following incubation with antitubulin antibodies.

Both of the above interpretations may be difficult to reconcile with the observation that antidynein antibodies appear to be able to bind only to the outer dynein arms, as mentioned above. It is difficult to see how antitubulin antibodies could penetrate the axoneme sufficiently to cause a complete inhibition of active bending by either of the above mechanisms. However, there is a third possible interpretation that does not suffer from this problem. This is a simple increase in the bending resistance of the outer-doublet microtubules, as a result of antibody binding to their exposed outer surfaces. Our measurements of the stiffness of antitubulin-inhibited flagella and the earlier measurements of CO_2 -inhibited flagella (21) indicate that there is no increase in the static bending resistance. However, the viscoelastic effect that we measured after incubation of flagella with antitubulin antibodies indicates that there is a high value of immediate bending resistance, which can also be described as a bending impedance. This impedance might be sufficient to prevent oscillatory bending at normal flagellar beat frequencies, while not preventing sliding disintegration. The viscoelastic response of the flagellum would, according to this interpretation, involve rearrangement of antitubulin antibodies bound to the surfaces of the microtubules, and should be independent of MgATP^{2-} concentration. Alternatively, antitubulin antibodies and CO_2 might cause an increase in static bending resistance of the outer-doublet microtubules that is highly nonlinear, such that it is not detected in our stiffness measurements, which impose relatively low values of curvature on the axoneme, but would be sufficient to prevent the axoneme from bending to a critical value of curvature required for sustained oscillation. Both the experiments with CO_2 (4) and those with antitubulin (data not shown) show that as incubation proceeds there is a relatively abrupt cessation of

oscillation when the bend angle falls below a critical value.

We have not seriously considered the possibility that antibodies inhibit by directly cross-linking adjacent microtubules, in part because this interpretation offers no understanding of the similarity between the effects of antitubulin antibodies and CO_2 . However, measurements of the rate of sliding of doublet microtubules during extrusion from protease-digested axonemes, using the techniques of Yano and Miki-Noumura (24), might be useful in evaluating this possibility.

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